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Laboratory Investigation

Seeding of Intravascular Stents With Genetically Engineered Endothelial Cells

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The use of intravascular stents may be limited by both local thrombosis and restenosis due to intimal proliferation. In an effort to provide solutions to these problems, we seeded stents with genetically engineered endothelial cells *in vitro*. Using retroviral-mediated gene transfer, we inserted the gene for either bacterial β -galactosidase or human tissue-type plasminogen activator (t-PA) into cultured sheep endothelial cells. The endothelial cells were seeded onto stainless steel stents and grown until the stents were covered. Expression of intracellular β -galactosidase and high level secretion of t-PA were demonstrated both before and after the transduced cells were seeded onto the stents. Eight stents were expanded by *in vitro* balloon inflation, with observation of the seeded endothelial layer both prior to and after expansion. Most of the endothelial cells remained on the stents after balloon inflation. We conclude that intravascular stents can be coated with a layer of genetically engineered endothelial cells that can be either specifically labeled or made to secrete high levels of a therapeutic protein. Much of the layer of genetically engineered cells remains after the expansion of the stent *in vitro*. *In vivo* implantation of stents coated with genetically engineered endothelial cells may allow 1) introduction of genetically engineered endothelial cells directly into the vascular wall and 2) improvement of stent function through localized delivery of anticoagulant, thrombolytic, or antiproliferative molecules. (*Circulation* 1989;80:1347-1353)

Intravascular stents have been in use for more than 20 years.^{1,2} Human studies, first reported in 1987,³ demonstrated the usefulness of stents in the treatment of iliac and coronary stenoses.^{4,5} Although the reported incidence of stent-related thrombosis in these early studies was low, more recently, other authors have recognized the clinical importance of early thrombotic stent closure.^{6,7} The risk of stent-related thrombosis appears to be an acute problem, temporally related to stent placement and presumably due to the thrombogenicity of the stent, the underlying arterial lesion, or both.⁸ Recent reviews by Schatz⁸ and King⁹ suggest that thrombogenicity is decreased as the stent surface is covered by regrowth of endothelium.

Van der Giessen et al¹⁰ addressed the problem of stent-related thrombogenicity by seeding freshly

harvested, human umbilical vein endothelial cells onto stainless steel stents. Complete stent coverage was achieved *in vitro*, prompting the authors to suggest that stents be seeded with autologous endothelium before placement *in vivo*, to reduce the risk of thrombosis. The implementation of such a protocol is made problematic by two considerations, both of which are well described in the literature on seeded vascular grafts. The first problem concerns the inability to differentiate among endothelial cells found covering a stent, those seeded on the stent prior to implantation from those growing onto the stent from the adjacent arterial wall.¹¹ The second consideration is how to ensure that the seeded endothelial cells express a net anticoagulant function because endothelium is capable of promoting both coagulation and thrombolysis.¹²

Retroviral-mediated gene transfer offers solutions to both the problem of cell identification and that of enhancement of thrombolytic activity. Transfer of the bacterial β -galactosidase gene into the seeded endothelial cells allows identification of implanted cells by a simple histochemical stain of the vascular wall.¹³ Expression of an inserted gene such as the human tissue-type plasminogen activator (t-PA) gene

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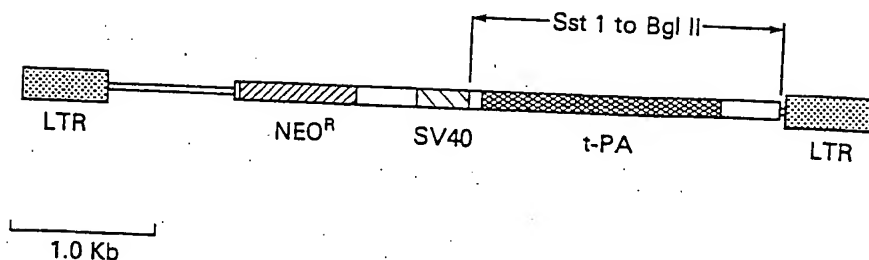


FIGURE 1. Diagram of B2NSt, retroviral vector used for transfer of human t-PA gene. LTR, long terminal repeat; NEO^R, neomycin phosphotransferase gene; SV40, SV40 early promoter; t-PA, human tissue-type plasminogen activator cDNA, 2.2 kb Sst I to Bgl II sequence. 5' and 3' untranslated regions of NEO^R and t-PA genes are left unshaded.

could permit high level secretion of t-PA, favoring a thrombolytic environment on the endothelial cell surface. Previous work in our laboratory has shown that endothelial cells can serve as targets for retroviral-mediated gene transfer.¹⁴ In the present study, sheep endothelial cells transduced with retroviral vectors containing either the bacterial β -galactosidase gene or the human t-PA gene were seeded on intravascular stents. We further evaluated the ability of these genetically engineered cells to continue to express the inserted genes after they were seeded onto the stents. We then expanded the stents with balloon catheters in vitro and examined retention of the cells on the stent surfaces.

Methods

Endothelial Cell Harvest and Culture

Endothelial cells were harvested from segments of adult sheep jugular vein, carotid artery, and femoral vein using the method of Jaffe et al.¹⁵ A total of four vessels from three sheep were used. Identification of harvested endothelial cells was confirmed by their cobblestone structure and by binding of the fluorescent ligand DiI-acetyl-LDL¹⁶ (Biomedical Technologies, Stoughton, Massachusetts). Cells were cultured on fibronectin-coated plastic culture dishes (1.0 $\mu\text{g}/\text{cm}^2$) (Collaborative Research, Bedford, Massachusetts) in M-199 (Biofluids, Rockville, Maryland) with 20% fetal calf serum (Hyclone Laboratories, Logan, Utah), 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B (Biofluids). Cells were passaged using trypsin-EDTA (Biofluids) digestion. Removal of sheep vessels was done according to protocols approved by the animal use committee of the National Heart, Lung, and Blood Institute.

Retroviral-Mediated Gene Transfer

A murine ecotropic packaging line capable of transmitting the β -galactosidase-containing "BAG" vector¹⁷ was a kind gift of Constance Cepko (Harvard University, Cambridge, Massachusetts). Supernatant from this packaging line was used to generate an amphotropic packaging line from PA-317 cells.¹⁸ A human t-PA cDNA (in plasmid pPA34'f)¹⁹ was generously provided by Sandra Degen (University of Cincinnati, Cincinnati, Ohio). This t-PA cDNA was used, through several subcloning steps, to construct a t-PA containing retroviral vector, B2NSt

(Figure 1), analogous in construction to the SAX vector.²⁰ The corresponding plasmid, based on the B2 plasmid,¹⁴ was transfected into GPE-86 cells,²¹ and supernatant from these cells was used to infect PA-317 cells, thereby, generating amphotropic packaging clones capable of transmitting the t-PA gene. Endothelial cells were transduced by incubation for 2 hours with supernatant-containing virions with the retroviral vector, along with 8 $\mu\text{g}/\text{ml}$ added Polybrene. After transduction, endothelial cells were selected in 0.2–0.4 mg/ml G-418 for at least 16 days. Duplicate cultures of cells from each vessel harvest were transduced simultaneously with either the t-PA- or β -galactosidase-containing retroviral vector and, then, cultured, passaged, and selected using identical procedures. In this manner, the t-PA- and β -galactosidase-transduced cells served as controls for one another in experiments involving either β -galactosidase activity or t-PA secretion.

Stent Seeding

Tubular slotted stainless steel 1.6-mm diameter stents⁸ (Johnson and Johnson Interventional Systems, Warren, New Jersey) were cut at the articulation, and each half was seeded with endothelial cells, using a modification of the method of Van der Giessen et al.¹⁰ A total of 10 stent segments were seeded. Stents were submerged in 100 $\mu\text{g}/\text{ml}$ human fibronectin for 15 minutes at 37° C and, then, transferred to polypropylene tubes containing a suspension of $6\text{--}10 \times 10^4$ endothelial cells in 0.8 ml culture medium. The tubes were placed in a 37° incubator containing 5% CO₂ and rotated 180° every 10 minutes for 2 hours, after which the stents and cell suspension were placed in wells of plastic tissue-culture dishes and additional culture medium added. Coverage of the stent surface was monitored both by phase-contrast microscopy and by incubation of the stents for 4 hours in medium containing DiI-acetyl-LDL followed by fluorescence microscopy.

Assays for Functions of Inserted Genes

The presence of the β -galactosidase gene product was determined by staining with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)¹³ of cells either on tissue-culture dishes or in situ on the stents. Levels of human t-PA were determined by enzyme-linked immunosorbent assay (ELISA) of tissue culture supernatants using a commercially

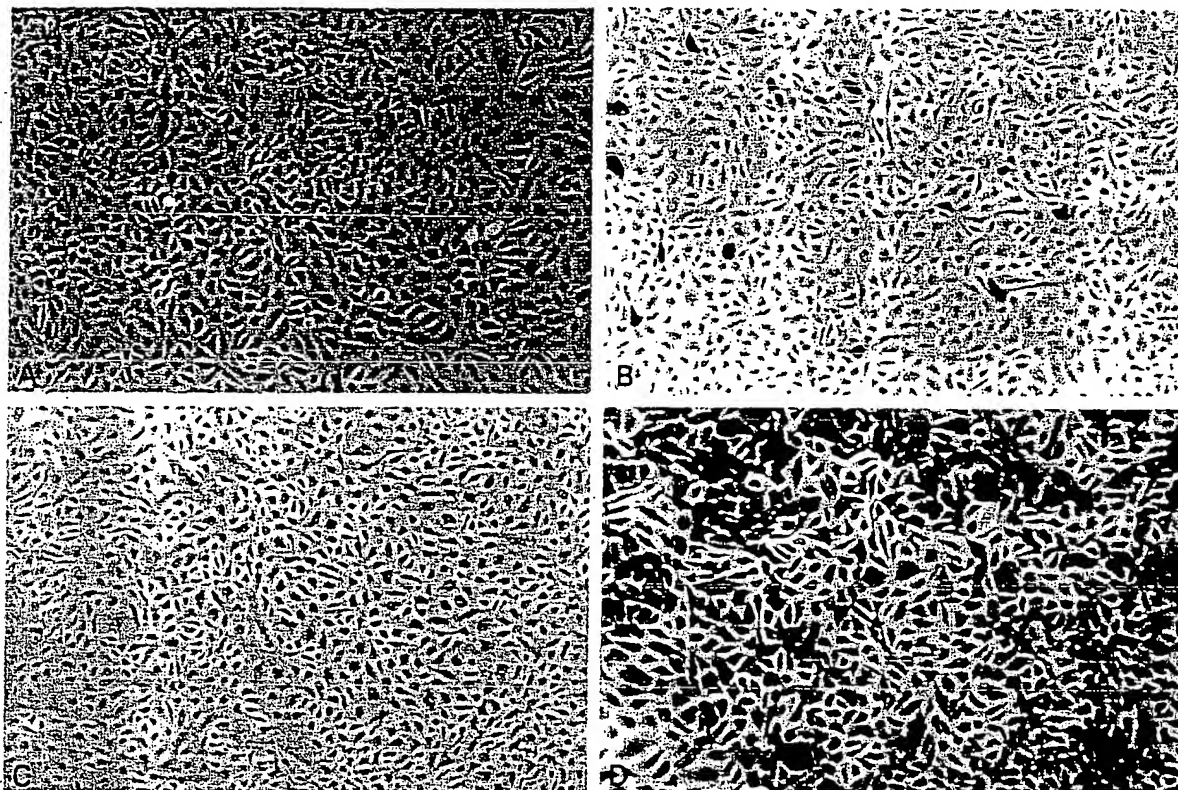


FIGURE 2. Photomicrograph depicting structure and X-Gal staining of sheep carotid artery endothelial cells. Panel A: Untransduced cells. Panel B: Cells transduced with β -galactosidase gene, X-Gal stained. Approximately 2–3% of the cells were transduced, consistent with the low (2×10^3 G-418 resistant cfu/ml) titer of our β -galactosidase producer line. Panel C: Cells transduced with t-PA gene, X-Gal stained. Panel D: Cells transduced with β -galactosidase gene, selected in G-418 to kill cells not expressing Neo^r gene, X-Gal stained. Approximately 80% of carotid artery cells stained blue following G-418 selection. Similar results were obtained for jugular vein and femoral vein endothelial cells.

available kit²² (American Diagnostica, New York, New York). Supernatant to be assayed was collected above confluent monolayers in 35-mm dishes, 48 hours after addition of 2 ml fresh medium. For measurement of t-PA secretion from a seeded stent, we transferred the stent to a new well containing fresh medium and, then, began a timed collection of culture medium. Harvested supernatant was centrifuged at 15,000g for 15 minutes to remove cellular debris, made 0.01% with Tween-80, and frozen at -70°C until assayed. The rate of t-PA secretion in nanograms per 10^6 cells per 24 hours was calculated using a confluent cell density of 3×10^4 cells/cm² of tissue culture plastic (data not shown).

Expansion of Seeded Stents and Visualization of Cells on Expanded Stents

Seeded stents were incubated in medium containing DiI-acetyl-LDL for 4 hours before expansion. The stents were visualized by fluorescence microscopy to confirm endothelial coverage and, then, manually placed over a deflated 3.0-mm diameter coronary angioplasty balloon catheter (Scimed Life Systems, Maple Grove, Minnesota). After balloon inflation to 4–6 atm, resulting in complete stent expansion, the balloon was deflated and the stents

were removed from the catheters and, again, viewed by fluorescence microscopy.

Results

Structure and DiI-Acetyl-LDL Binding

Transduced sheep endothelial cells retained their cobblestone structure (Figure 2) and their ability to bind the fluorescent ligand DiI-acetyl-LDL (Figure 3). No difference in structure was detectable between those cells that had been transduced with the β -galactosidase vector and those that were transduced with the t-PA vector (Figures 2B and C).

X-Gal Staining

Only cells in cultures transduced with the β -galactosidase gene exhibited deep blue cytoplasm on staining with X-Gal (Figures 2B and D). After G-418 selection, most of the β -galactosidase-transduced cells stained deep blue with X-Gal (Figure 2D).

t-PA Secretion

Endothelial cells from all four vessels, when transduced with the t-PA vector, secreted immunoreactive t-PA. Rates of t-PA secretion (mean \pm SD

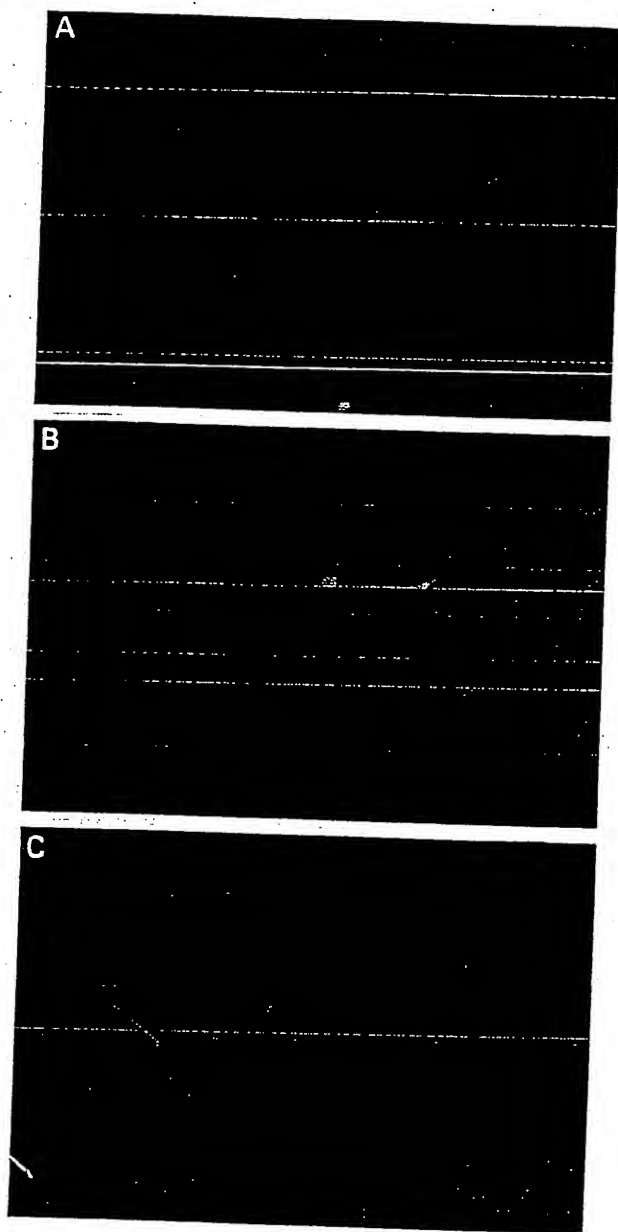


FIGURE 3. DiI-acetyl-LDL binding to transduced endothelial cells on stents. Panel A: Subconfluent stent; note that stent material does not autofluoresce. Panel B: Confluent monolayer covers stent. Panel C: Stent after expansion. Two bare areas are visible (arrows), but most of monolayer is intact.

of duplicate tissue culture wells, expressed as ng/ 10^6 cells/24 hr) were femoral vein, 370 ± 8 ; carotid artery, 660 ± 240 ; jugular vein 1, 230 ± 6 ; jugular vein 2, $2,200 \pm 18$. t-PA production by the β -galactosidase-transduced cells was below the lower limit of sensitivity of the assay (i.e., less than 5 ng/ 10^6 cells/24 hr) in all of the supernatants tested (Figure 4).

Seeding of Stents With Endothelial Cells Expressing Recombinant Genes

Fluorescence microscopy of six of the seeded stents confirmed complete coverage of the visible

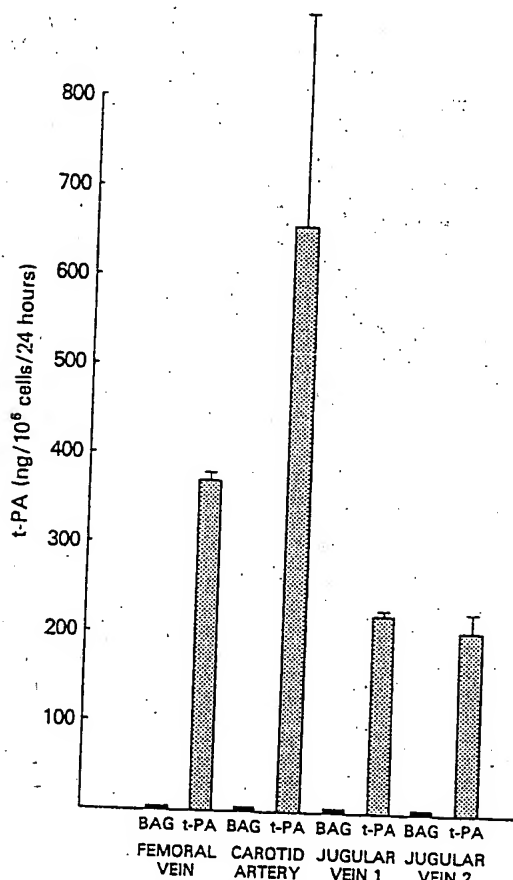


FIGURE 4. Bar graph of tissue-type plasminogen activator (t-PA) secretion by transduced sheep endothelial cells. Endothelial cells from four separate harvests of blood vessels were transduced with t-PA or β -galactosidase gene and then subjected to G₄₁₈ selection for a minimum of 16 and a maximum of 23 days. With one exception, ELISA was performed on supernatants from cells in duplicate tissue-culture dishes. Femoral vein endothelial cells transduced with β -galactosidase gene were represented by a single tissue culture dish. Data are presented as mean \pm SD of duplicate dishes. The supernatants of β -galactosidase-transduced cells all produced less than 5 ng/ 10^6 cells/24 hr of immunoreactive t-PA (lower limit of detection of assay). Triplicate ELISA wells were used to determine concentration of t-PA in supernatant of t-PA-transduced cells.

stent surfaces (Figures 3B and C). When eight stents seeded with either β -galactosidase- or t-PA-transduced endothelial cells were stained with X-Gal, the stents covered with β -galactosidase-carrying cells turned blue, whereas the stents covered with t-PA-secreting cells did not (Figure 5). Measurement of human t-PA levels from the cell culture medium surrounding the stents confirmed that t-PA was being secreted only by the t-PA-transduced endothelial cells. Three stents seeded with t-PA-transduced endothelial cells secreted 6.3, 4.8, and 2.6 ng t-PA/24 hr (Table 1). t-PA secretion by the β -galactosidase-transduced cells on each of

TABLE 1. Secretion of t-PA From Stents Seeded With β -Galactosidase (BAG)- or t-PA-Transduced Endothelial Cells

	t-PA secretion (ng/24 hr)			Mean \pm SD
	SCAEC	SJVEC-1	SJVEC-2	
t-PA	6.3	4.8	2.6	4.6 \pm 1.8
BAG	<2.5	<0.75	<0.75	—

SCAEC, sheep carotid artery endothelial cells; SJVEC-1,2, sheep jugular vein endothelial cells, harvests 1 and 2; t-PA, tissue plasminogen activator.

three stents, if present, was below the limit of detection of the assay.* To check the internal consistency of our results, we used the measured t-PA secretion from each of three lines of transduced cells both before and after they were seeded onto stents to calculate the surface area of the stents. This calculation is based on the assumption that the density of the cells and the rate of t-PA secretion do not change when the cells are on the stents. We calculated a stent surface area (mean \pm SD) of $48 \pm 19 \text{ mm}^2$, not significantly different from the manufacturer's value of 42 mm^2 (personal communication, Johnson and Johnson Interventional Systems, Warren, New Jersey). Our overestimation of the stent surface area may be due to either continued t-PA secretion by cells that have divided and fallen off the stent or piling up of the cells in the interstices of the stent (Figures 3B and C).

Cellular Retention After Balloon Inflation

Four stents covered with DiI-acetyl-LDL-stained endothelial cells were expanded using balloon catheters and immediately viewed with a fluorescence microscope. Near-complete retention of the cells on the exterior surfaces of all four stents was confirmed (Figure 3C). X-Gal staining of stents carrying β -galactosidase-transduced cells permitted evaluation of cellular retention on all surfaces after balloon inflation. The stents were viewed with a dissecting microscope, and cellular retention on all surfaces was estimated. A total of eight expanded stents were observed after X-Gal staining, four covered with β -galactosidase-transduced endothelial cells. We found that much of the interior lumen surface of the stents was free of cells after balloon inflation but that the cellular layer on the exterior and lateral stent-strut surfaces was largely intact (Figure 5).

*The differences in assay sensitivities in Table 1 and Figure 5 are due to differences in the amount of culture medium in which the tissue-culture monolayers and stents were incubated. Because the assay measures t-PA concentration, the use of a lower volume of medium will increase the sensitivity of the assay to detect mass of t-PA. In all cases, we assume, according to the manufacturer's instructions, that the lower limit of sensitivity of the assay is the detection of 1.5 ng/ml t-PA. By placing seeded stents in low volumes of medium and collecting the medium after a 48-hour incubation, we were able to measure concentrations of secreted t-PA in the range of 10–30 ng/ml, well above the lower limit of assay sensitivity.

Discussion

Retroviral-mediated gene transfer has been used to insert genes into a variety of cell types including hematopoietic cells, fibroblasts, neural cells, hepatocytes, keratinocytes,^{23,24} and, most recently, endothelial cells.¹⁴ The goal of these gene-transfer studies has usually been the cure of a genetically based deficiency disease. Sanes et al,¹³ however, applied retroviral gene transfer to the study of cell lineage in the developing embryo, and the first use of retroviral-mediated gene transfer in humans will use the inserted neomycin resistance gene to track cells in vivo.²⁵ The goals of our gene transfer study differ in that we are using gene insertion to enhance the function of otherwise normal endothelial cells that are then used to improve the clinical efficacy of an implantable biomedical device. We have succeeded in coating stainless steel intravascular stents with endothelial cells, producing either a marker (β -galactosidase) or a therapeutic protein (t-PA). Given the thrombotic problems associated with intravascular prosthetic devices, including grafts and arti-

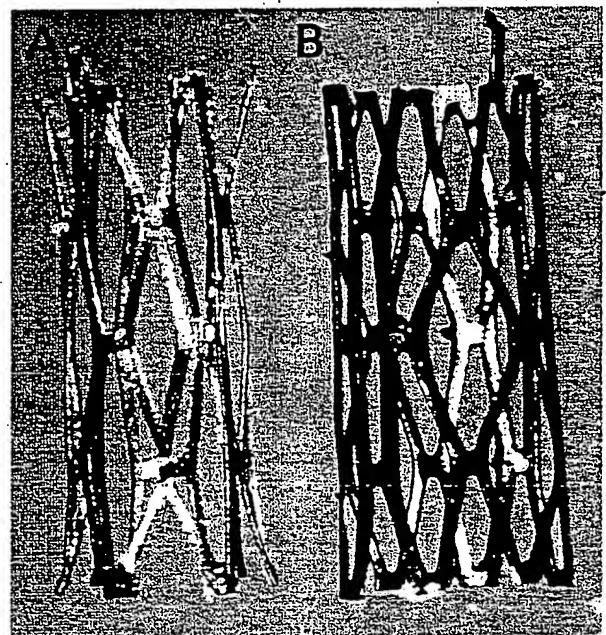


FIGURE 5. Photographs of X-Gal stain of seeded stents. After balloon expansion, stents were stained with X-Gal. A: Stent seeded with t-PA-transduced cells. B: Stent seeded with β -galactosidase-transduced cells.

cial hearts, the potential applications of this technology are substantial.

Endothelial cells will not grow on bare metal; therefore, the application of a substrate is necessary before cell seeding. This layer is thought to be provided in vivo through the deposition of a layer of fibrin and thrombus.⁸ We used a fibronectin coating in vitro to allow endothelial cell adhesion to the stents. Because a fibronectin coating can increase platelet adherence,²⁶ it is possible that, when many of the luminal cells are removed from the stent during balloon inflation, exposed fibronectin can lead to thrombus formation. This possibility was addressed by Seeger and Klingman²⁷ in an in vivo study of fibronectin-coated, endothelial cell-seeded vascular grafts in dogs. Platelet adherence to fibronectin-coated grafts was higher than adherence to control grafts, but there was no decrease in graft patency. Analogous in vivo stent studies will be required to address the thrombotic risks of fibronectin-coated stents.

Retention of seeded cells after stent expansion was excellent on the external and lateral surfaces but poor on the internal lumen surface. This is presumably due to mechanical effects of balloon expansion. Use of stent designs that do not depend on balloon expansion, such as the spring-loaded stent,⁸ may improve luminal cell retention. The success of our stent-seeding protocol in vivo is clearly dependent on the ability of the remaining cells to adhere to either the stent or the adjacent vascular wall and to proliferate overexposed stent and vessel-wall surfaces. A significant advantage of our ability to genetically label endothelial cells with the β -galactosidase gene is that these cells and their progeny can be unequivocally identified at a later time by histochemical staining. In this manner, we will be able to definitively evaluate the ability of intravascular stents to act as scaffolds for the localized introduction of genetically engineered endothelial cells. Wilson et al²⁸ and Nabel et al²⁹ recently reported encouraging data on the ability of implanted transduced endothelial cells to survive and proliferate in vivo. These authors used seeded vascular grafts in dogs and in vivo seeding of the pig aorta, respectively, to demonstrate survival and proliferation of implanted β -galactosidase-transduced endothelial cells for at least 4–5 weeks. This length of time is probably far more than enough for endothelial coverage of the stent surfaces because our endothelial-cell in vitro doubling time is only 2–3 days (data not shown). In vivo trials, however, will be required to establish that this is the case.

We demonstrated very high levels of secretion of human t-PA from transduced sheep endothelial cells. The levels we measured are the highest ever reported for secretion of t-PA from cultured endothelium, and are up to two orders of magnitude greater than those previously measured from cultured human endothelial cells.^{30–32} The concentrations of t-PA that we achieve in vitro are far greater than that

required for activation of plasminogen and consequent fibrinolysis.³³

In vivo, we expect that cells carried on the stents will repopulate, at most, a small area of arterial surface bathed by a large volume of blood. The concentrations of t-PA we achieve in vitro will certainly not be duplicated in vivo. Our goal, however, is not to produce systemic levels of t-PA but, rather, to produce a local thrombolytic environment. High level secretion of t-PA adjacent to a forming clot may permit t-PA to be concentrated through the high affinity binding of t-PA to fibrin.³⁴ In this manner, fibrinolytic activity would be directed to microthrombi beginning to form on the stent surface or downstream, thus, preventing the formation of occlusive thrombi. Hergreuter et al³⁵ demonstrated in a rabbit model that locally but not systemically administered t-PA could abort thrombus formation on a highly thrombogenic inverted artery. Intravascular stents are far less thrombogenic than is an inverted vessel, and it is possible that localized delivery of nanogram quantities of t-PA will result in sufficient thrombolytic activity to prevent stent-related thrombotic events.

Restenosis of stented arterial segments secondary to intimal hyperplasia also limits the long-term benefits of intravascular stents.⁸ The implantation of genetically engineered endothelial cells on stent surfaces offers a potential means of preventing intimal hyperplasia because implanted endothelial cells would be in direct contact with the intima and could be engineered to secrete proteins capable of inhibiting intimal growth. Until the molecular basis of intimal hyperplasia is more clearly defined, however, this application will remain theoretical.

Without examining the effect of t-PA-gene insertion on the expression of heparin, plasminogen activator inhibitor, thrombomodulin, protein S, and other endothelial cell procoagulant and anticoagulant molecules, one cannot be certain of the net effect of t-PA-gene insertion on endothelial cell fibrinolytic activity. Although such studies are potentially interesting, their in vitro results cannot be directly transferred to an in vivo environment. Extrapolation from subcultured isolated endothelial cells growing on plastic in tissue-culture medium and serum to in vivo endothelium bathed in blood and tissue fluid in a vascular wall is not without risk.^{36,37} Given the ongoing clinical concerns of stent-related arterial occlusion and the availability of animal models for stent-related thrombosis,^{3,38} we feel it is appropriate to proceed with in vivo studies to determine whether intravascular stent function can be improved by seeding stents with genetically engineered endothelial cells.

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